Influence of sulfite on growth, slime, and fluorescent pigment formation by *Pseudomonas aeruginosa*[1]

3795

SAMUEL A. PALUMBO

Eastern Regional Research Laboratory, U.S. Department of Agriculture, Philadelphia, Pennsylvania 19118

PALUMBO, S. A. 1973. Influence of sulfite on growth, slime, and fluorescent pigment formation by *Pseudomonas aeruginosa*. Can. J. Microbiol. **19**: 505–511.

Substitution of sulfite for sulfate in the defined pyocyanine medium of Frank and DeMoss 1959 allowed formation of fluorescent pigments and slime by *Pseudomonas aeruginosa* NRRL B-4014. This formation of fluorescent pigments was both pH and iron dependent. The unadjusted medium (pH 8.25) containing sulfite and Fe^{3+} allowed both growth and fluorescent pigment formation. Growth and fluorescent pigment formation were observed from a pH of 9.0 down to a pH of 7.5. At pH 7.5, the concentration of HSO_3^- is 6×10^{-4} M, and this ion appears to be the active agent in inhibiting growth below pH 7.5. When the medium was adjusted to pH 7.0, neither fluorescent pigment formation nor growth was observed. The acid used for pH adjustment did not influence the minimum pH for growth. In the presence of small amounts of iron (ca. 1×10^{-6} M Fe^{3+}), the organism shifted from a blue to a yellow-green fluorescent pigment. Cultural conditions such as temperature and nutrients which supported growth also supported slime formation as well as fluorescent pigment formation.

PALUMBO, S. A. 1973. Influence of sulfite on growth, slime, and fluorescent pigment formation by *Pseudomonas aeruginosa*. Can. J. Microbiol. **19**: 505–511.

La substitution du sulfite pour le sulfate dans le milieu défini pyocyanine de Frank et DeMoss 1959 permet la formation de pigments fluorescents et de mucilinage par *Pseudomonas aeruginosa* NRRL B-4014. Cette formation de pigments fluorescents est dépendante du fer et du pH. Le milieu non ajusté (pH 8.25) contenant le sulfite et le Fe^{3+} permet la croissance et la formation de pigments fluorescents. La croissance et la formation de pigment fluorescent sont observées du pH 9.0 jusqu'au pH 7.5. A pH 7.5, la concentration de HSO_3^- est 6×10^{-4} M, et cet ion apparaît être l'agent actif de l'inhibition de la croissance à pH plus faible que 7.5. Lorsque le milieu est ajusté à pH 7.0, ni la formation de pigment fluorescent ni la croissance sont observées. L'acide utilisé pour l'ajustement du pH n'influence pas le pH minimum pour la croissance. En présence de faibles quantités de fer (ca. 1×10^{-6} M Fe^{3+}), l'organisme change d'un pigment fluorescent bleu à un pigment jaune-vert. Les conditions culturales telles que la température et les nutrients qui supportent la croissance, supportent aussi bien la formation de mucilinage que celle de pigments fluorescents. [Traduit par le journal]

Introduction

During a recent study of the influence of sulfur source and iron on pigment and slime formation by *Pseudomonas aeruginosa*, it was observed that when sulfite was substituted for sulfate as the source of sulfur added to the defined pyocyanine medium of Frank and DeMoss (6), *P. aeruginosa* formed fluorescent pigments instead of the pyocyanine, two other phenazines, and pyorubrin, which are usually formed (15). This formation of fluorescent pigments was both iron and pH dependent. The unadjusted pH of sulfite-containing media was 8.25, and this pH allowed both growth and fluorescent pigment formation. However, when the pH of sulfite-containing medium was adjusted to 7.0, no growth occurred and fluorescent pigment was not found. *Pseudomonas aeruginosa* formed two different fluorescent pigments when sulfite was added to the medium; when iron was omitted from sulfite-containing media, a blue fluorescent pigment was formed, while with the addition of iron, a yellow-green fluorescent pigment was formed. Therefore, it was decided to investigate (*a*) the pH dependency of growth, slime, and fluorescent pigment formation in sulfite-containing media, (*b*) the substrates and cultural conditions which will support production of slime and fluorescent pigments in sulfite-containing media, and (*c*) the role of iron in fluorescent pigment formation by this organism.

[1]Received September 22, 1972.

Materials and Methods

Organism and Medium

Pseudomonas aeruginosa NRRL B-4014 was grown in modified pyocyanine medium of Frank and DeMoss (6). This medium (sulfite pyocyanine or SP broth) contains DL-alanine, 10 g; glycerol, 25 g; Na_2SO_3, 12.6 g (0.1 M); $MgCl_2 \cdot 7H_2O$, 4.02 g; K_2HPO_4, 0.139 g; ferric citrate, 0.1 g per liter. Other carbon and nitrogen sources were

substituted at the same concentration as the compound they were replacing. In one experiment, a solution of $FeCl_3 \cdot 6H_2O$ replaced the ferric citrate. The pH was adjusted with 1 N HCl or other acid or with 1 N NaOH; without adjustment, the starting pH was 8.25. Incubation was stationary at 30C because shaking prevented slime formation (4). Growth (turbidity) was indicated as either plus (+) or minus (−) except where it was quantitated as below.

Pigment Analysis

Fluorescent pigments were measured in an Aminco Bowman Spectrophotofluorometer[2] at two settings; the blue pigment was excited at 365 nm and its emission quantitated at 440 nm, and the yellow-green pigment was excited at 410 nm and its emission quantitated at 525 nm. The slime and cells were separated from the broth by centrifugation (16 300 × *g* for 10 min in the cold) and the fluorescent pigments were measured as stated. The pH was measured after centrifugation and in most instances was the same as the starting pH before growth.

Influence of pH on Growth, Slime, and Fluorescent Pigment Formation

Media containing sulfite with and without iron were adjusted with NaOH or HCl to various pH values from 9.0 to 7.0 in 0.2-unit increments and inoculated. After 7 days incubation, the culture was blended for ca. 15 s at high speed in a Waring Blendor to break the slime network and permit separation of the cells from the slime material. The blended culture was centrifuged in the cold (0C) at 1935 × *g* for 1 h. The cells were washed once with distilled water, recentrifuged, and dried overnight at 110C. The slime was then precipitated from the supernatant by the addition of an equal volume of absolute ethanol and centrifuged at 16 300 × *g* for 15 min in the cold. The slime was washed with ethanol : water (1:1) and recentrifuged, and then dried overnight at 110C. Slime was also measured by the slime index as described previously (15). With this method, cultures showing sliminess (a slime index greater than 1.0) were designated as positive. The fluorescent pigments were then quantitated as described above.

Inhibition by Bisulfite

The possibility that the inhibition of growth of *P. aeruginosa* in SP broth adjusted to pH 7.0 was due to the action of bisulfite was investigated by adding amounts of bisulfite to the broth equivalent to those formed when a 0.1 M sulfite solution was adjusted to pH 8.0, 7.5, and 7.0 (18). Growth (turbidity) was evaluated simply as plus (+) or minus (−).

Iron Gradient

To determine the role of iron in the formation of the blue and the yellow-green fluorescent pigments, graded amounts of $FeCl_3 \cdot 6H_2O$ were added to broth containing sulfite. The amounts of the two fluorescent pigments were then quantitated.

[2]Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Temperature and Nutrient Variation

To separate slime and fluorescent pigment formation from growth, temperature of incubation and carbon and nitrogen sources were varied. Since slime production is a fundamental characteristic of *P. aeruginosa* (8), slime production in SP broth and in Haynes' medium (8) was further studied. It was of interest to investigate the components of each broth which are required for slime production. This study was performed by omitting and substituting various components in these two media. To define further the role of sulfur source and iron in the formation of pigments by *P. aeruginosa*, the different pigments formed in the presence and absence of iron and sulfur with these variations were quantitated.

Results

Iron Gradient

The amounts of the two fluorescent pigments formed by *P. aeruginosa* when incubated in SP broth with and without iron are given in Fig. 1; the starting pH was 8.25 and did not change during growth. Because the Fe^{3+} concentrations used ranged from 0.74×10^{-3} M to 0.74×10^{-12} M, the Fe^{3+} concentration was plotted on log scale. Higher concentrations of iron were not tried because of the formation of insoluble $Fe(OH)_3$ at alkaline pH values. Figure 1 shows that (1) there is an iron concentration (0.74×10^{-6} M) at which the organism will form both fluorescent pigments, and (2) the low level of Fe^{3+} (0.74×10^{-6} M) at which the organism will shift from the blue to the yellow-green fluorescent pigment. The low level of iron at

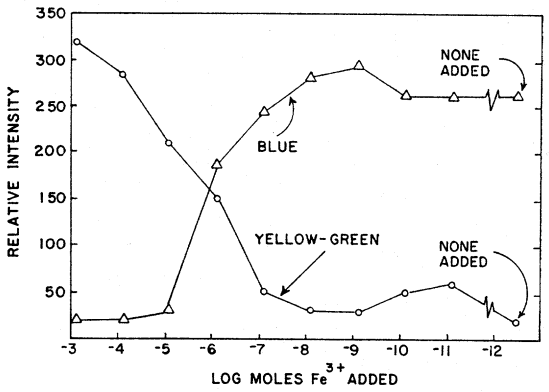

FIG. 1. The influence of Fe^{3+} concentration on the production of the blue and the yellow-green fluorescent pigments by *Pseudomonas aeruginosa* NRRL B-4014. The basal medium used was SP broth with ferric citrate omitted; iron was a solution of $FeCl_3 \cdot 6H_2O$. (Starting pH was 8.25 and did not change during growth.)

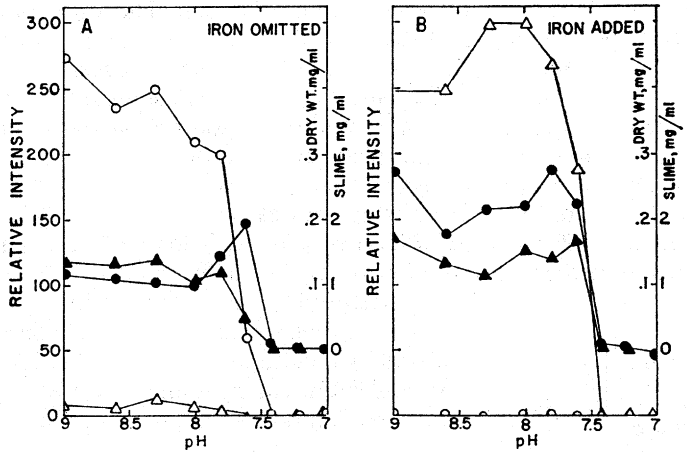

FIG. 2. The influence of pH and iron (0.1 g ferric citrate per liter) on growth, fluorescent pigment, and slime formation by *Pseudomonas aeruginosa* NRRL B-4014: growth as dry wt. (●), slime as dry wt. (▲), blue fluorescent pigment (○), and yellow-green fluorescent pigment (△).

which the organism shifts from the blue to the yellow-green fluorescent pigment indicates that the yellow-green pigment is a secondary metabolite.

pH Gradient

It was observed early in the study that the formation of fluorescent pigments was pH dependent when sulfite was the added sulfur source. This pH dependency was investigated by determining the influence of pH on growth, slime, and fluorescent pigment formation by the organism. The pH values are the starting values, but these did not change during growth. These data are presented in Fig. 2; Fig. 2A presents data obtained when iron was omitted from SP broth, and Fig. 2B data when iron (0.1 g ferric citrate per liter) was added to this medium. These figures show that growth of *P. aeruginosa* was inhibited between 7.6 and 7.4. Slime and fluorescent pigment formation were affected at pH 7.6. Growth, slime, and fluorescent pigment formation were influenced at about the same pH values, regardless of whether iron was absent or present. However, the organism grew better and produced more slime when iron was added to the medium.

The influence of pH on the growth of *P. aeruginosa* in SP broth was further investigated by using different acids to adjust the pH of the medium. SP broth was adjusted to pH 8.0, 7.5, and 7.0 with HCl, HNO_3, H_2SO_4, and H_3PO_4, and acetic, lactic, and succinic acids, respectively, and observed for growth (turbidity) after 7 days incubation at 30C. Growth was observed at both pH 8.0 and 7.5 with all acids and no growth occurred at pH 7.0 with any of the acids. Thus, it was probably not any of the salts which were formed when the pH of the medium was lowered to pH 7.0 that caused the inhibition of growth.

Bisulfite Inhibition

The data presented above suggest that the addition of acid to SP broth inhibited the growth of *P. aeruginosa* when the pH of the medium was below 7.5. This inhibition was due probably

TABLE 1

The influence of bisulfite on the growth of *Pseudomonas aeruginosa* NRRL B-4014. The basal broth contained DL-alanine, glycerol, K_2HPO_4, and $MgCl_2 \cdot 7H_2O$

Moles[a] HSO_3^-	pH of broth with HSO_3^- added	Growth
2×10^{-4}	6.90	+
6×10^{-4}	6.70	+
1.9×10^{-3}	6.35	−
	pH of broth	
—	6.90	+
—	6.70	+
—	6.35	+

[a]These concentrations of HSO_3^- correspond to the amount of HSO_3^- formed from 0.1 M Na_2SO_3 at pH 8.0, 7.5, and 7.0, respectively.

to the conversion of small quantities of sulfite to bisulfite by the acid. Using the pH-bisulfite data of Rehm and Wittmann (16), concentrations of bisulfite, corresponding to the amounts formed when SP broth is adjusted to pH 8.0, 7.5, and 7.0, were tested for their inhibitory action against *P. aeruginosa* (Table 1). Since bisulfite is itself slightly acidic, control broths adjusted to the same pH values as the different concentrations of bisulfite were also investigated for their inhibitory action. These data are given in Table 1.

TABLE 2

The influence of temperature, sulfite, and iron on slime and fluorescent pigment formation by *Pseudomonas aeruginosa* NRRL B-4014. The basal broth contained DL-alanine, glycerol, K_2HPO_4, and $MgCl_2 \cdot 7H_2O$

Temperature, °C	Addition[a]	Slime	Blue	Yellow-green
20, 30, 37	None	−	+	+
20, 30, 37, 41	Fe^{3+}	−	−	−
20, 30, 37, 41	$SO_3^=$	+	+	−
20, 30, 37	Fe^{3+} and $SO_3^=$	+	−	+
41	None	−	−	−
41	Fe^{3+} and $SO_3^=$	+	−	−

[a] Fe^{3+} concn., 0.1 g ferric citrate per liter. $SO_3^=$ concn., 12.6 g Na_2SO_3 per liter.

TABLE 3

The influence of carbon and nitrogen source and iron on slime and fluorescent pigment formation by *Pseudomonas aeruginosa* NRRL B-4014 at 30C. The basal broth contained K_2HPO_4, $MgCl_2 \cdot 7H_2O$, and Na_2SO_3

C and N source	Iron	Slime	Blue	Yellow-green
Glycerol and DL-alanine[a]	−	+	+	−
Glycerol and DL-alanine[b]	+	+	−	+
NaCitrate and DL-alanine[c]	−	+	+	+
Glucose and DL-alanine[d]	−	+	−	−
Glycerol and L-asparagine[e]	−	−	+	−
Glycerol and proteose-peptone[f]	−	−	+	+

[a] Other substrates giving this pattern: NaSuccinate and DL-alanine, mannitol and DL-alanine, NaK tartrate and DL-alanine, DL-alanine as C and N source, glycerol and L-proline, glycerol and DL-isoleucine, glycerol and L-alanine, glycerol and L-leucine, glycerol and acetamide.
[b] Other substrates giving this pattern: glycerol and L-proline, glycerol and L-asparagine, glycerol and DL-isoleucine, glycerol and L-alanine, glycerol and hydroxy-L-proline, glycerol and acetamide, glycerol and L-leucine, DL-alanine and NaCitrate, DL-alanine and NaSuccinate, DL-alanine and dextrose, DL-alanine and mannitol, DL-alanine and NaK tartrate, DL-alanine and NaAcetate, DL-alanine as C and N source, L-asparagine as C and N source, proteose-peptone as C and N source.
[c] Other substrates giving this pattern: NaAcetate and DL-alanine, glycerol and Na L-glutamate, glycerol and DL-aspartic acid.
[d] Only these substrates gave this pattern.
[e, f] Only these substrates with and without glycerol gave these patterns.

Temperature and Nutrient Variation

The effects of incubation temperature, different carbon and nitrogen sources on slime, and fluorescent pigment formation, independent of cellular growth by *P. aeruginosa*, were investigated. Four temperatures over the organism's normal growth range were used along with the omission / addition of iron or sulfite or a combination of both. These data are presented in Table 2. Growth occurred with all variables. These data illustrate that cultural conditions of temperature, iron, and sulfite can be found which will separate slime and fluorescent pigment formation from growth.

The effects of various carbon and nitrogen sources as replacement for the glycerol and DL-alanine of SP broth on slime and fluorescent pigment formation by *P. aeruginosa* were investigated; the starting pH values with the various substrates were adjusted to 8.25. Substrates were selected on the basis of their ability to support growth of *P. aeruginosa* (3, 6, 19) or to support fluorescent pigment formation (7, 9, 10, 11, 16). The slime-pigment patterns obtained are presented in Table 3. The first two patterns are typical of most substrates tested. The third pattern is characteristic of only four substrate combinations and probably represents low-level iron contamination of these substrates. The last three patterns were obtained only with their

TABLE 4

The influence of individual components in SP broth and Haynes' medium on slime and fluorescent pigment formation by *Pseudomonas aeruginosa* NRRL B-4014 at 30C

Medium / broth	Addition[a]	Slime	Blue	Yellow-green
Haynes'	gluc-try	+	+	+
"	gly-try	−	+	+
"	gluc-ala	+	+	+
"	gluc-try-FeCit	+	−	−
"	gluc-try-$SO_3^=$	+	−	−
"	gluc-try-FeCit-$SO_3^=$	+	−	−
SP	gly-ala	+	−	+
"	gluc-ala	+	−	+
"	gly-try	−	−	−
"	gluc-try	+	−	−

[a] gluc = potassium gluconate, 40 g/liter; ala = DL-alanine, 10 g/liter; try = tryptone, 1.5 g/liter; FeCit = ferric citrate, 0.1 g/liter; gly = glycerol, 25 g/liter; $SO_3^=$ = Na_2SO_3, 12.6 g/liter.

respective substrates. The pattern observed suggests a complex mode of control of both slime and fluorescent pigment formation by the organism.

The ability of the various components of Haynes' broth and SP broth to support slime and fluorescent pigment formation after 7 days incubation at 30C is shown in Table 4. In both Haynes' medium base and the SP broth base, potassium gluconate appears to be the component which supported slime formation. Similar data were obtained for Haynes' medium when incubated at 20, 37, and 41C.

Discussion

Many media have been formulated to stimulate fluorescent pigment formation in bacteria (7, 9, 10, 11, 16) which depend on various combinations of carbon and nitrogen substrates to stimulate fluorescent pigment formation. The medium modification presented here to stimulate fluorescent pigment formation by *P. aeruginosa* depended only on substitution of sulfite for sulfate in the defined pyocyanine medium of Frank and DeMoss (6). The apparent stimulation of this ion functioned with all substrates and cultural conditions tested except as shown in Tables 2 and 3. Iron plays a unique role in fluorescent pigment formation by *P. aeruginosa.* When iron was added to SP broth, the organism formed a yellow-green fluorescent pigment, while with its omission, the organism formed a blue pigment. The controversy over the role of iron in media for detecting fluorescent pseudomonads was discussed previously (15).

Osawa *et al.* (14) observed that differences in tone and fluorescence of a fluorescent pigment fraction from a *P. aeruginosa* culture were dependent upon the pH of the solution. The possibility existed that the fluorescence observed in the present study at the two different wavelengths might be the same pigment, and that differences in the pH of the medium could give the appearance of two different pigments. The data suggest otherwise. When sulfite (0.1 M) was substituted for sulfate in the original pyocyanine medium of Frank and DeMoss (6), the pH was raised to 8.25 and remained there during growth and assay. The sulfite provided considerable buffering capacity and the organism was unable to alter this high pH during growth. The data in Fig. 1 show that, depending on the presence or absence of iron, two different fluorescent pigments were formed. Furthermore, it was observed previously (15) that the yellow-green fluorescent pigment was not an iron chelate of the blue fluorescent pigment. The influence of pH on fluorescent pigment formation was then investigated (Fig. 2). Both iron and pH were varied. The fluorescent pigments formed are a function of iron. From pH 9 to 7.5, the blue fluorescent pigment was formed in the absence of iron, and in the same pH range, the yellow-green fluorescent pigment was formed in the presence of iron. In both cases, pH values below 7.5 were not considered because no growth occurred. In SP broth, iron rather than pH appeared to be the factor governing the appearance of the fluorescent pigments.

The data presented in Fig. 1 support the secondary metabolite nature of the yellow-green fluorescent pigment (21). Whether the blue pigment is also a secondary metabolite whose production is under the control of another metal was not investigated. However, considering the large number of pigments that are secondary metabolites and that the yellow-green pigment may represent only a slight structural modification of the blue fluorescent pigment, the blue fluorescent pigment is probably a secondary metabolite.

Totter and Moseley (20) observed that production of fluorescin by *P. aeruginosa* was inversely proportional to the log of the iron concentration. Since they gave no spectral data for fluorescin, there is no way of determining whether fluorescin is similar to the yellow-green fluorescent pigment described here. However, if the two pigments are similar, then the data presented support their findings. Lenhoff (13) has reported an inverse relationship between iron and fluorescin production by *Pseudomonas fluorescens.*

The addition of iron to SP broth increases the growth yield (dry weight) of *P. aeruginosa.* This increased growth may be reflected in the higher iron requirement of the organism. Knight (12) reported that *P. aeruginosa* has a higher iron requirement than *Escherichia coli.* This was not tested, but increases in the iron content of SP broth might yield further increases in growth. Yield of slime material also increased

with the addition of iron, but this was probably related to the increase in the amount of cells.

The pH dependency of growth of *P. aeruginosa* in SP broth is related to the formation of small quantities of bisulfite when the pH of the medium is lowered from 8.25 to 7.0. The quantity of bisulfite formed at pH 7.0 is quite small, 1.9×10^{-3} M. However, bacteria are more sensitive to bisulfite (HSO_3^-) than are yeasts and molds (17, 18). Rahn and Conn (17) reported that the growth of *E. coli* is inhibited by 10 mg bisulfite/100 ml or ca. 1×10^{-3} M, and this is about the same quantity that inhibits *P. aeruginosa*.

Sulfite stimulated formation by *P. aeruginosa* of a slime which was found to be protein with no carbohydrate (15). The ash content of this slime was not determined, but that from other strains of *P. aeruginosa* was reported to be 8 to 9% (5), 10% (1), and 13 to 15% (2). The absolute requirement for sulfite in slime formation could be reflected in the sulfur content of the ash from this slime material. Eagon (4) observed that *P. fluorescens* (organism was really *P. aeruginosa*) needed an alkaline reaction (pH 8.0) and (or) a high concentration of potassium or sodium for slime production on sugars. SP broth as formulated has both a pH of 8.25 and a high concentration (0.1 M) of Na ion.

The data on temperature influence on slime and fluorescent pigment formation in SP broth and in Haynes' medium (Tables 2 and 4) indicate that the activity of the organism in SP broth at all temperatures is definitely component related, whereas in Haynes' medium, the pattern is the same at all temperatures. In complete SP broth, no pigment is formed at 41C while in Haynes' medium both fluorescent pigments are formed. Certain precursors needed for fluorescent pigment formation at 41C may be supplied in the complex Haynes' medium and may be lacking in the simple defined SP broth.

The data presented here support the general findings of Frank and DeMoss (6) concerning substrates and pyocyanine production. Substrates that support growth of *P. aeruginosa* also support fluorescent pigment and slime formation. The data in Table 2 indicate that under certain cultural conditions, e.g., temperature, growth can be separated from slime and fluorescent pigment formation. However, the observations in Table 3 suggest that if growth occurs in SP broth, *P. aeruginosa* will also produce slime and fluorescent pigments.

1. Bartell, P. F., T. E. Orr, and B. Chudio. 1970. Purification and chemical composition of the protective slime antigen of *Pseudomonas aeruginosa*. Infect. Immun. **2**: 543–548.
2. Brown, M. R. W., J. H. S. Foster, and J. R. Clamp. 1969. Composition of *Pseudomonas aeruginosa* slime. Biochem. J. **112**: 521–525.
3. Burton, M. O., B. A. Eagles, and J. J. R. Campbell. 1947. The amino acid requirement for pyocyanin production. Can. J. Res., Sect. C, Bot. Sci. **25**: 121–128.
4. Eagon, R. G. 1956. Studies on polysaccharide formation by *Pseudomonas fluorescens*. Can. J. Microbiol. **1**: 673–676.
5. Eagon, R. G. 1962. Composition of an extracellular slime produced by *Pseudomonas aeruginosa*. Can. J. Microbiol. **8**: 585–586.
6. Frank, L. H., and R. D. DeMoss. 1959. On the biosynthesis of pyocyanine. J. Bacteriol. **77**: 776–782.
7. Georgia, F. R., and C. F. Poe. 1932. Study of bacterial fluorescence in various media. II. The production of fluorescence in media made from peptones. J. Bacteriol. **23**: 135–145.
8. Haynes, W. C. 1951. *Pseudomonas aeruginosa*—its characterization and identification. J. Gen. Microbiol. **5**: 939–950.
9. Jamieson, M. C. 1942. Requisites for the recognition of the blue-green *Pseudomonas*. Sci. Agric. **22**: 401–409.
10. King, E. O., M. K. Ward, and D. E. Raney. 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44**: 301–307.
11. Klinge, M. 1959. *Pseudomonas fluorescens*, ein Boden- und Wasserkeim. I. Physiologie und Identifizierung. Arch. Mikrobiol. **33**: 1–24.
12. Knight, S. G. 1951. Mineral metabolism. *In* Bacterial physiology. *Edited by* C. H. Werkman and P. W. Wilson. Academic Press, New York.
13. Lenhoff, H. 1963. An inverse relationship of the effects of oxygen and iron on the production of fluorescin and cytochrome *c* by *Pseudomonas fluorescens*. Nature (London), **199**: 601–602.
14. Osawa, S., E. Yabuuchi, Y. Narano, M. Nakata, Y. Yakawa, K. Takashina, T. Tanabe, and H. Tomono. 1964. Pigment production by *Pseudomonas aeruginosa* on glutamic acid medium. Studies on fluorescence I. Jap. J. Microbiol. **8**: 115–123.
15. Palumbo, S. A. 1972. Role of iron and sulfur in pigment and slime formation by *Pseudomonas aeruginosa*. J. Bacteriol. **111**: 430–436.
16. Paton, A. M. 1959. Enhancement of pigment production by *Pseudomonas*. Nature (London), **184**: 1254.
17. Rahn, O., and J. E. Conn. 1944. Effect of increase in acidity on antiseptic efficiency. Ind. Eng. Chem. **36**: 185–187.
18. Rehm, H.-J., and H. Wittmann. 1962. Beitrag zur Kenntnis de antimikrobiellen Wirkung der schwefligen Säure. I. Übersicht uber einflussnehmende Faktoren auf die antimikrobielle Wirkung der

schwefligen Säure. Z. Lebensm.-Unters. Forsch. **118**: 413–429.
19. STANIER, R. Y., N. J. PALLERONI, and M. DOUDEROFF. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. **43**: 159–271.
20. TOTTER, J. R., and F. T. MOSELEY. 1953. Influence of the concentration of iron on the production of fluorescin by *Pseudomonas aeruginosa*. J. Bacteriol. **65**: 45–47.
21. WEINBERG, E. D. 1970. Biosynthesis of secondary metabolites: roles of trace metals. *In* Advances in microbial physiology. Vol. 4. *Edited by* A. H. Rose and J. F. Wilkinson. Academic Press, London. pp. 1–44.